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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Examiner	: Susan E. Fernandez	
Serial No.	: 10/695,574	
Filed	: October 28, 2003	
Inventor(s)	: Denis Barritault	Docket No.: 1003-DIV-01
	: Jean-Pierre Caruelle	
Title	: BIOCOMPATIBLE POLYMERS, PROCESS	Confirmation No.: 4857
	: FOR THEIR PREPARATION AND	
	: COMPOSITIONS CONTAINING THEM	

DECLARATION OF DULCE PAPY-GARCIA UNDER 37 C.F.R. 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I hereby declare as follows:

1. I, Dulce PAPY-GARCIA, am Pharmacist, PhD in Organic Chemistry, and full University Professor in Sugar Chemistry and Biochemistry. A copy of my curriculum vitae is attached hereto as Exhibit A.

2. I have read and understood US patent application serial no. 10/695,574 (the "Application"), and have read the Office Action concerning the Application mailed on July 5, 2005.

3. The July 5, 2005 Office Action states that the Application supposedly does not contain sufficient teaching to allow one of ordinary skill in the art to make and use AXY polymers as defined by the Application claims, except for those specifically disclosed in the Application. Thus, I understand that the Examiner believes that the Application does teach one skilled in the art how to make and use AXY polymers where A is -(O-CH_2-CH_2-CO)- or glucose; X is -COOH or –COO^-Na^+; and Y is -CO-CH_2-CHOH-CH_2-SO_3H, -CO-CH_2-CHOH-CH_2-SO^{3-} Na^+, -SO_3H or -$SO^{3-}Na^+$. The July 5, 2005 Office Action also states that the Application supposedly does not adequately describe all AXY polymers encompassed by the Application claims, but again describes only those polymers which are specifically disclosed. I consider one of ordinary skill in the art is someone with a PhD degree in Organic or sugar chemistry applied to polymer chemistry, and/or who has about 5 years relevant working experience in Organic or Sugar Chemistry applied to Polymer Chemistry.

4. Concerning to how to make AXY polymers, I consider that the information contained in the Application is sufficient to allow one of ordinary skill in the art to make the claimed AXY polymers and not only those specifically disclosed in the Application. The information contained in the application should be sufficient to prepare polymers at least of the general formula: A.sub.aX.sub.xY.sub.y.

5. The teachings of the Application do convey to one of ordinary skill in the art how to make and use the full range of polymers encompassed by the Application claims. For example, Examples 1 and 2 on pages 22 and 29 of the Application, respectively, show the synthesis of AXY polymers in which A is -(O-CH_2-CH_2-CO)- or glucose. One of ordinary skill in the art would have understood, as of the Application filing date, that other sugars could also be used in the disclosed synthesis scheme with little or no modification to produce the claimed AXY polymers. The substitution of other sugars into the synthesis scheme could be readily accomplished with only routine experimentation by one of ordinary skill in the art.

6. Concerning the group A in the AXY polymers: The application describes: "A as *a monomer, which can he identical or different, selected from the group consisting of sugars, esters, alcohols, amino acids and nucleotides.*" I consider that with the information contained in

the application, together with their own knowledge, one of ordinary skill in the art will be able to prepare polymers AXY in where A is a sugar unit, (corresponding to any sugar unit, not only glucose, but galactose, xylose, manose, etc.) in a polysaceharide and where A can be different sugars, as in the saccharidic moiety of glycoproteins or in other glycans. I consider that the information contained in the application is enough to allow one of ordinary skill in the art to prepare polymers AXY in where A can also be a molecule bearing alcohols groups, as polyalcohols.

7. Concerning the group X: The application describes: *"X represents a carboxyl bearing group (-R--COO--R', in which R is a bond or an aliphatic hydrocarbon chain, optionally branched and/or unsaturated, and which can contain one or more aromatic rings except for benzylamine and benzylamine stalfonate, and R' represents a hydrogen atom or a cation".* I consider that with the information contained in the application, together with their own knowledge, one of ordinary skill in the art will be able to make polymers AXY in where X represents a carboxyl bearing group (--R--COO--R') in which R is an alkyl (R = -[CH_2]n , where n≥l), an allyl (R = -CH=CH[CH_2]n, where n≥l), aryl, linear or branched groups and R' is a hydrogen atom or a cation.

8. Concerning the group Y: The application describes: *"Y represents a sulfate or sulfonate group bonded to monomer A and is contained within a group according to one of the following formulas: --R--O-SO.sub.3--R', --R- -SO.sub.3--R , --R--SO.sub. 3--R', in which R is a bond or an aliphatic hydrocarbon chain, optionally branched and/or unsaturated, and which can contain one or more aromatic rings except for benzylamine and benylamine sulfonate, and R' represents a hydrogen atom or a cation,".* I consider that with the information contained in the application, together with their own knowledge, one of ordinary skill in the art will be able to make polymers AXY in where Y represents a sulfate group bonded to monomer A according to the following formula: --R--SO.sub.3--R` in which R is a bond, an alkyl (R = -[CH_2]n, where n≥l), allyl (R = - CH=CH[CH_2]n, where n≥l), aryl, linear or branched groups

9. Furthermore, concerning the additional group Z: The application describes: *"Z is a substance different from X and Y, which confers on the polymer additional solubility or*

lipophilic properties, supplementary biological or physicochemical properties, or a therapeutic or diagnostic agents. " The Application states also that *"Z can be identical or different, and selected from the group consisting of amino acids, fatty acids, fatty alcohols. ceramides or derivatives thereof and nucleotide addressing sequences.* " I consider that with the information contained in the application, together with their own knowledge, one of ordinary skill in the art will be able to make polymers AXYZ in where Z can be identical or different, and selected from the group consisting of amino acids or derivatives thereof.

10. Thus, one skilled in the art would understand from their own knowledge and the teachings of the Application that substitution of the X, Y, and Z components as claimed would not alter the reaction chemistry as disclosed throughout the Application, and in Examples 1 and 2 in particular. The Application therefore contains sufficient teaching to allow one of ordinary skill in the art to make and use the AXY polymers claimed in the Application.

11. I consider that one of ordinary skill in the art would also be able to immediately envision all the various AXY polymers encompassed by the Application claims, and would understand that such polymers could be made by the disclosed synthetic schemes or by employing routine techniques known to those of ordinary skill in the art. For example, the applicants state that the AXY polymers can contain -(O-CH_2-CH_2-CO)- or any sugar as the A component, and one of ordinary skill in the art would understand that any sugar could be readily substituted into the disclosed synthetic schemes without significantly altering the reaction chemistry. Given the Application's disclosure, in particular Examples 1 and 2, one of ordinary skill in the art could also readily envision the specific AXY polymers formed by using sugars other than glucose in the disclosed reaction schemes. Likewise, substitution of other groups for the X and Y components as discussed above is disclosed in the Application, and one of ordinary skill in the art would understand that these groups could be readily substituted into the claimed AXY polymers without significantly altering the reaction chemistry, and could readily envision the resultant AXY polymers. Thus, one of ordinary skill in the art would understand that the Applicants had possession of the entire claimed range of AXY polymers as of the Application filing date.

12. The July 5, 2005 Office Action also states that the Application supposedly does not provide one of ordinary skill in the art with a reasonable expectation that administration of the claimed AXY polymers would successfully treat or reduce fibrosis *in vivo*. The Application presents *in vitro* data which shows that the claimed AXY polymers inhibit the growth of fibrosis-forming cells such as smooth muscle cells, fibroblasts or hepatic cells, and restore the quantity and quality of collagen produced by such cells under conditions expected to induce fibrosis (*e.g.*, radiation) to that of control cells. See, *e.g.*, Examples 12 and 13 and Figs. 23-26 of the Application. One of ordinary skill in the art would interpret these data to mean that administration of the claimed AXY polymers would produce the same effect on fibrosis-forming cells *in vivo*. Thus, the *in vitro* data presented in the specification is sufficient to give one of ordinary skill in the art a reasonable expectation that the claimed polymers could be used to reduce or treat fibrosis *in vivo*. This is supported by the fact that in the last 20 years, many *in vitro* results have been used as a proof of efficacy *in vivo* and are now considered as standards by one of ordinary skill in the art and regulatory authorities. Concerning safety, regulatory authorities now request *in vitro* assays in the field of genotoxicity (Ames, TK Locus etc.). For cosmetic products, animal assays are forbidden in Europe under a new regulation. One of ordinary skill in the art therefore understands that demonstration of safety and efficacy must be provided through *in vitro* assays. For example, efficacy for anti wrinkle agents is supplied with *in vitro* assays on skin fibroblast showing increases of collagen synthesis. The use of *in vitro* data to ascertain *in vivo* effects is thus well established for one of ordinary skill in the art. In the field of growth factors and their uses as wound healing agents or for colony stimulating factors or for interleukin, the efficacy of the growth factor for *in vivo* uses are measured routinely by *in vitro* assays. In the case of the present invention, the AXY polymers of the invention are selected on the basis of stabilizing, potentiating and protecting growth factors such as FGFs or TGF beta. This means to one of ordinary skill in the art that AXY regulates the activity of the growth factors which are involved in the fibrosis formation and will reduce fibrosis *in vitro*. It is therefore a reasonable expectation for one of ordinary skill in the art that the claimed polymers will obtain the same effect *in vivo*.

13. The undersigned declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and

further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 15/05/2006 Signature: 

Exhibit A

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DIPLOMAS AND CERTIFICATES:

1996	**PhD Sciences (Organic Synthesis, Sugar Chemistry)** Tokushima Bunri University, Tokushima, Japan.
1993	**Master in Pharmaceutical Sciences (Organic Chemistry)** Tokushima Bunri University, Tokushima, Japan.
1990	**Diploma on the study of the Japanese Language and Culture** University of Foreign studies of Osaka, Japan.
1989	**Pharmacist** School of Chemistry-Pharmacy-Biology University of Michoacan, Mexico.
1989	**Awarder as First Young Pharmaceutical Chemist** Clinical Chemistry Society, Mexico.

RESEARCH AND PROFESSIONAL POSITIONS:

1996-1997	**Post-Doct Position (Biopolymer Sciences)** Ecole Nationale Supérieure de Chimie, Toulouse, France.
1997-2001	**Project Manager and Researcher** ValbioFrance Company, Paris, France.
2001-2005	**Assistant Professor (Biological Chemistry)** University Paris 12, Faculty of Science and Technologies, France.
2001-2005	**Full Professor (Biochemistry and Glycosciences)** University Paris 12, Faculty of Science and Technologies, France.
From 2004	**Head of the Research Group ATIP 'Heparanome. Structure-Function' Created by the French National Center of Scientific Research (CNRS)** University Paris 12, Faculty of Science and Technologies, France.
From 2003	**Member of the 'NeuroPrion European Excellence Network'**

Collagen Synthesis by Human Intestinal Smooth Muscle Cells in Culture

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Growth kinetics and collagen production were determined in smooth muscle cells isolated from human jejunum and maintained in cell culture. Collagen synthesis increased during the first 15 days in culture at a time when the rate of cell proliferation was maximal. When confluent, these cells produced significantly more collagen than human dermal fibroblasts cultured under identical conditions. The smooth muscle cells required daily replenishment of ascorbate for maximal collagen synthesis. The types of collagen produced by human intestinal smooth muscle cells in culture were the same as those collagens extracted from strictured human bowel (types I, III, and V). These findings suggest that collagen production by human intestinal smooth muscle cells has a role in the repair as well as the fibrosis of the gastrointestinal tract.

The pathogenesis of stricture formation in the gastrointestinal tract is unknown. This narrowing of the lumen of the bowel is characterized by an accumulation of collagen in the intestinal wall (1). In the past it has been assumed that this collagen was produced by fibroblasts. Our initial studies of collagen isolated from inflamed intestine have suggested, however, that this protein was synthesized by intestinal smooth muscle cells and not by fibroblasts (2).

To investigate further the possible role of human intestinal smooth muscle (HISM) cells in intestinal stricture formation, these cells were isolated, maintained in culture (3), and analyzed for their collagen production. The results demonstrated that HISM cells produce significant amounts of collagen in vitro, and that this process was enhanced by cell proliferation and by ascorbate. In addition, the types of collagens synthesized by these cells in vitro were the same as those extracted from strictured human bowel. Synthesis of large quantities of collagen is a function of these cells that has heretofore not been recognized. Because such a large fraction of protein production was committed to the synthesis of collagen (6%–12%), we suggest that this function is a specialized one and may have a major role in the repair of the injured gastrointestinal tract. Intestinal fibrosis and subsequent stricture formation are possible complications of this repair process.

Materials and Methods

Cell Isolation and Culture

Smooth muscle cells were isolated from human jejunum by collagenase digestion of slices of muscularis propria as previously described (3; American Type Culture Collection, Catalog No. CRL 1692, Rockville, Md.). For comparative studies, human aortic smooth muscle cells were isolated in similar fashion by collagenase digestion of strips of media from thoracic and infrarenal aortas obtained from cadaver organ donors. As a second reference cell line, fibroblasts were grown from explants of normal human dermis. Smooth muscle cell cultures were incubated for 3 wk in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS) after which they were trypsinized and replated for use in experiments. Fibroblasts were used in the fifth or sixth passage.

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The authors thank K. Eastburn and N. Gotschalk for technical assistance, O. Nuckols for secretarial assistance, Dr. Chris Clark for his many helpful suggestions regarding the isolation and identification of collagens by electrophoresis, Dr. Harvey Sugerman for his help in procuring specimens, and Dr. Lindblad and Dr. Wolf for their helpful discussions of this work.

0016-5085/87/$3.50

Abbreviations used in this paper: FCS, fetal calf serum; HISM, human intestinal smooth muscle.

Determination of Collagen Synthesis

Effect of cell growth. After passage from primary culture, HISM cells were incubated in Dulbecco's modified Eagle medium containing 10% FCS and were refed daily with medium containing ascorbate (0.1 mM). At various time intervals, cells were incubated for 5 h in serum-free medium containing L-[5-^{3}H]proline (Schwarz/Mann, 4 μCi/ml) and fresh ascorbate. The cells and medium were scraped from the plates, combined, and heated to 90°C to destroy protease activity (4). The radioactive protein was removed from unincorporated [^{3}H]proline by repeated precipitation with 5% trichloroacetic acid at 4°C and was then dissolved in 0.2 N NaOH. Collagen was specifically solubilized by digestion with purified bacterial collagenase (5), after which the noncollagenous protein was reprecipitated with 5% trichloroacetic acid–0.5% tannic acid. Radioactivity was determined in the supernatants containing collagen-derived peptides and the pellets containing noncollagenous protein. The deoxyribonucleic acid content of samples was determined fluorometrically (6).

Effect of ascorbate. Human intestinal smooth muscle cells were fed daily with medium containing fresh ascorbate (0.1 mM) for either 2, 4, 6, or 8 days before incubation with L-[5-^{3}H]proline. Cells not supplemented with ascorbate were given fresh medium. After 8 days in culture, collagen synthesis was determined as described above.

Quantitation of Human Intestinal Smooth Muscle Cell Proliferation

Cell proliferation was quantitated in cultures identical to, and incubated in parallel with, those cultures used for determination of collagen synthesis. Cells were removed from the culture dishes by treatment with 0.1% trypsin for 10 min and then counted using a hemocytometer.

Determination of Collagen Types Synthesized by Human Intestinal Smooth Muscle Cells In Vitro as Compared With Collagen Isolated From Human Intestinal Stricture

After passage from primary culture, HISM cells were grown for 10 days in Dulbecco's modified Eagle medium supplemented with 10% FCS. Cells were then incubated for 6 h in serum-free medium containing ascorbate (0.1 mM), [^{14}C]proline (0.5 μCi/ml), L-[5-^{3}H]proline (25 μCi/ml), L-[5-^{3}H]glycine (25 μCi/ml) (ICN, Irvine, Calif.), and β-amino propionitrile (0.1 mg/ml). Protease inhibitors [ethylenediaminetetraacetic acid (20 mM), N-ethylmaleimide (8 mM), and phenylmethylsulfonyl fluoride (1 mM)] and 0.5 M acetic acid were then added. After 18 h at 4°C, the combined material was dialyzed against deionized water and then 0.4 M NaCl–0.1 M Tris. Chymotrypsin (0.3 mg/ml) was added for 6 h at 10°C after which N-tosyl-L-phenylalanine chloromethyl ketone (0.1 mg/ml) was added overnight to stop the reaction (7). The soluble material was then dialyzed against 0.5 M acetic acid (4°C). Collagen was precipitated in NaCl (4.5 M) and then resolubilized in acetic acid. The collagen bands were separated by polyacrylamide slab gel electrophoresis (8) using a urea buffer (8 M), 3% stacking gel, and 5% separating gel. The dried gel was then exposed to photographic film.

For analysis of collagen types present in strictured bowel, collagen was extracted from the surgically resected strictured ileum of a patient with Crohn's disease. The sample was minced, extracted with 0.5 M acetic acid and pepsin (0.1 mg/ml, 4°C, 6 h), neutralized with sodium hydroxide to inactivate the pepsin, and then dialyzed against 0.5 M acetic acid. Salt precipitation of collagen and polyacrylamide slab gel electrophoresis were performed as for the radioactive samples.

Results

Effect of Proliferation on Human Intestinal Smooth Muscle Cell Collagen Synthesis

Human intestinal smooth muscle cells proliferated rapidly for the first 15 days in culture (Figure 1). The incorporation of [^{3}H]proline into collagen, measured between days 5 and 15 of this growth period, increased more than twofold. In contrast, incorporation of the isotope into noncollagen protein decreased 50% over this time period. Collagen production was maximal at a time when the cells were approaching confluence on day 15. From days 15 to 25 in culture, the rate of cell proliferation decreased markedly. During this latter time period, both collagen and noncollagen protein production decreased.

Effect of Ascorbate on Human Intestinal Smooth Muscle Cell Collagen Synthesis

Because ascorbate has been demonstrated to be a critical cofactor for maximal expression of fully hydroxylated collagen by fibroblasts (9,10), we next examined the effect of this cofactor on collagen expression by HISM cells. Culture medium was supplemented with ascorbate for 2, 4, 6, or 8 days before measurement of collagen. Increases in absolute collagen synthesis (counts per minute per plate) and relative collagen synthesis (percentage of total protein synthesized) were both dependent on the duration of ascorbate supplementation (Figure 2). Neither noncollagen protein synthesis (Figure 2) nor cell proliferation (data not shown) was affected by ascorbate supplementation.

To determine whether the requirement of HISM cells for ascorbate was modified by the concentration of FCS in the medium, cultures were fed daily with medium containing 0.5%, 3%, or 10% FCS, with and without ascorbate. Relative collagen synthesis by HISM cells increased 50% with daily addition of

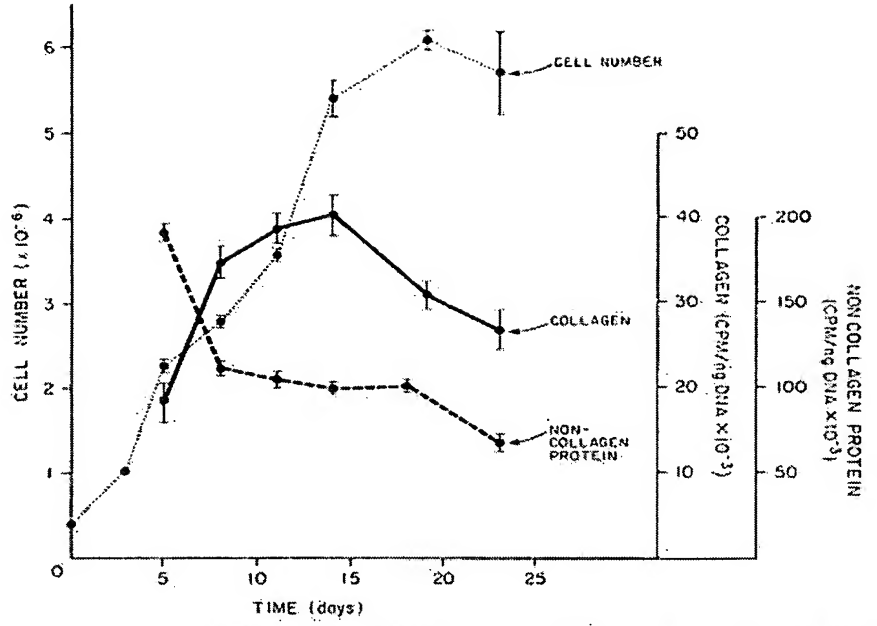

Figure 1. Effect of cell growth on collagen synthesis by HISM cells. After passage from primary culture, cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% FCS and ascorbate (0.1 mM). Medium was replaced daily. At each time interval, cells were pulsed with [^{3}H]proline for determination of incorporation into collagen and noncollagen protein. Values are the mean ± SEM, n = 4. Cell numbers were determined in identical culture dishes.

ascorbate, irrespective of the concentration of FCS present (data not shown).

Comparison of Collagen Synthesis by Human Intestinal Smooth Muscle Cells, Human Aortic Smooth Muscle Cells, and Fibroblasts

Smooth muscle cells from both intestine and aorta produced significantly more collagen compared to dermal fibroblasts (Table 1). When these data were calculated on the basis of deoxyribonucleic acid content, the HISM cells produced ~10-fold more collagen than did dermal fibroblasts. Because this increase could be due to differences in uptake and cell pools of proline in the two different cell types, collagen synthesis relative to total protein synthesis was also compared. This method of analysis has been established as a valid means of eliminating these possible differences between cell types, and is based on the assumption that a common amino acid pool is used for both collagen and noncollagen protein synthesis in the same cell (4). When calculated on this basis, collagen synthesis by HISM cells was twice that of fibroblasts.

Identification of the Collagen Types Synthesized by Human Intestinal Smooth Muscle Cells In Vitro

Autoradiograms of radioactive collagen produced by HISM cells in culture demonstrated that the major collagen type synthesized by HISM cells in vitro was type I (Figure 3). Type III collagen was also produced in relatively large amounts as evidenced by the movement of a major band into the area of the α_1(I) band following pretreatment with dithiothreitol. Type V collagen was produced but in relatively smaller amounts, as shown by the presence of the α_1(V) band which required enhancement by prolonged exposure. These collagen types were identical to those identified in collagen extracted from strictured bowel. A gel representative of 10 samples is shown in Figure 4.

Discussion

These studies have demonstrated that smooth muscle cells isolated from normal human intestine produce large amounts of collagen in vitro and that this expression of collagen production can be mod-

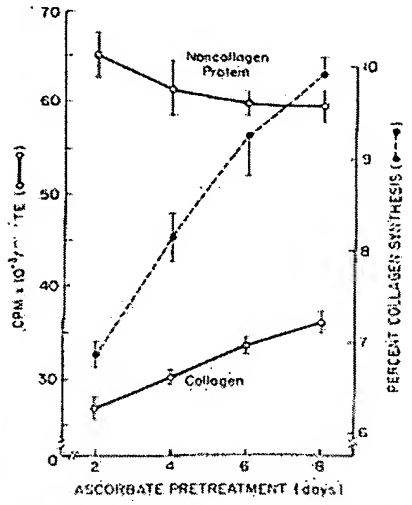

Figure 2. Effect of ascorbate pretreatment on collagen synthesis by HISM cells. Cells were fed daily with medium containing fresh ascorbate (0.1 mM) for either 2, 4, 6, or 8 days before incubation with [2,5-³H]proline. Cells not fed with ascorbate were given fresh medium. Collagen synthesis was determined after 8 days in culture. Because ascorbate showed no effect on cell proliferation (data not shown), absolute collagen and noncollagen protein synthesis are expressed as the amount of [³H]proline incorporated as a function of total number of cells.

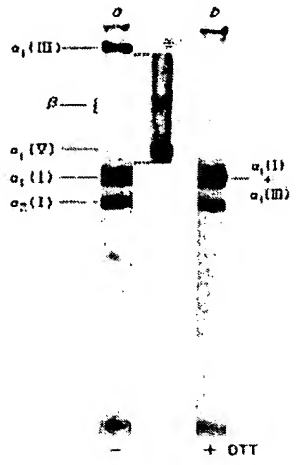

Figure 3. Collagen types synthesized by HISM cells in vitro. Cells were grown in medium containing 10% FCS for 10 days after which they were pulsed with isotopes as described in Methods. Collagen was extracted with acetic acid after digestion with chymotrypsin. *a*. Unreduced sample (–) showed α_1 type III, α_2 type V, and α_1 and α_2 type I collagen chains. *This portion of the gel was exposed for a longer time period to better demonstrate the presence of the α_1[V] band. β-Chains of type I collagen were also visible upon longer exposure. *b*. Sample reduced by 200 mM dithiothreitol before application to the gel. The disappearance of the type III band at the top of the gel was associated with an increase in the density of the α_1 type I band and is characteristic of type III collagen. Identification of the collagens was confirmed by their comigration with appropriate standards.

Table 1. *Collagen Synthesis by Human Intestinal Smooth Muscle Cells Compared to Human Aortic Smooth Muscle Cells and Human Dermal Fibroblasts*

Cell type	Collagen (cpm/μg DNA)	Noncollagen (cpm/μg DNA)	Relative collagen synthesis (%)
Fibroblast	2.9 ± 0.1	11.0 ± 0.3	4.6 ± 0.1
HASM	34.7 ± 4.9*	45.8 ± 7.0*	12.5 ± 0.3*
HISM	20.2 ± 0.5*	38.8 ± 0.6*	0.8 ± 0.3*

DNA, deoxyribonucleic acid; HASM, human aortic smooth muscle; HISM, human intestinal smooth muscle. Values are mean ± SEM, $n = 6$. *Significantly different from corresponding values for fibroblasts ($p < 0.01$, by one-way analysis of variance using a two-tailed t-test). The three cell types were analyzed under identical conditions of culture. Collagen synthesis was determined after 14 days in culture when cells were confluent. Relative collagen synthesis is expressed as a percentage of total protein synthesized after correction for the enriched proline and hydroxyproline content of collagen compared to that of other proteins (4).

ulated by the proliferative state of the cells and the presence of ascorbate.

The fibroblast has in the past been thought of as the cell responsible for both repair and fibrosis and has thus been extensively studied. More recently, work has been reported on collagen production by arterial smooth muscle cells (11–14), and these cells are now thought to be responsible for the synthesis of collagen in atherosclerotic plaque (15). When collagen synthesis of confluent HISM cells was compared with that of human aortic smooth muscle cells and human dermal fibroblasts cultured under identical conditions, both the HISM cells and human aortic smooth muscle cells produced significantly more collagen relative to total protein than the fibroblasts (Table 1). These results suggest that collagen can be a major synthetic product of HISM cells. Ultrastructural studies of chronically inflamed (16) and hypertrophic (17) bowel have demonstrated

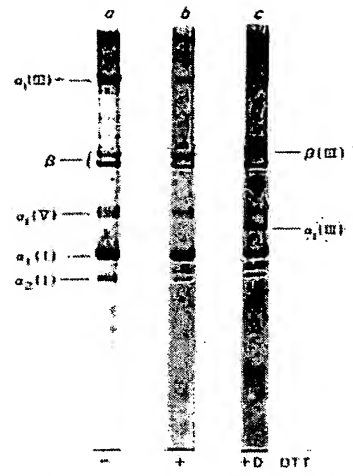

Figure 4. Collagen types extracted from strictured bowel. Collagen was extracted from the surgically resected strictured ileum of a patient with Crohn's disease as described in Methods. *a*, Unreduced sample (–) showing α_1 type III, α_1 type V, and α_1 and α_2 type I chains. *b*, Sample reduced by exposure to 200 mM dithiothreitol before application to the gel. The type III band diminished in density and moved into the area of the α_1(I) chain which became more dense. *c*, The presence of type III collagen was clearly demonstrated by the delayed reduction technique where dithiothreitol (650 mM) was added after 90 min of electrophoresis. The α_1 type III collagen band became visible between the α_1V band and the α_1(I) band. A β-chain of type III collagen was also visible (lane c). Identification of the collagens was confirmed by their comigration with appropriate standards. These findings have been observed in 10 of 10 samples of strictured bowel extracted in identical fashion.

increased numbers of collagen fibrils in close association with smooth muscle cells. The data presented here substantiate the inference made by the ultrastructural studies that intestinal smooth muscle cells can have a major role in the production of collagen in the intestinal wall under pathologic conditions.

Collagen synthesis by HISM cells required daily supplementation with ascorbate for maximum expression (Figure 2). In addition, the stimulation of collagen production by ascorbate was independent of the concentration of FCS in the medium. Both of these observations are in contrast to studies of avian tendon fibroblasts (10), which showed that only 48 h of ascorbate supplementation was required for maximal collagen synthesis and that the requirement for ascorbate could only be demonstrated in the presence of low (0.5%) concentrations of serum. The findings from the present study, therefore, suggest that collagen expression by HISM cells is enhanced by ascorbate in ways that may be distinct from the process observed in fibroblasts.

Collagen production by HISM cells increased markedly while cells were proliferating, reached a maximum when the cells achieved confluence, and then decreased (Figure 1). This increase in collagen expression during cell proliferation has been observed by others in studies of arterial smooth muscle cells (11,12). In addition, arterial smooth muscle cells, when stimulated by serum, have been shown to switch from a contractile to a synthetic state (13). The stimuli that induce this change and its reversal at confluence are unknown but probably depend on signals between cells, and between cells and the extracellular matrix. It is possible that cells, while proliferating at a low density, have a requirement to synthesize extracellular matrix. Our findings in this study of HISM cell collagen production are consistent with those done on arterial cells. The increase in collagen production that we have documented during proliferation of HISM cells would coincide with a change to a synthetic state. This increase in collagen synthesis appeared to be specific for collagen because there was a concomitant reduction in noncollagen protein synthesis (Figure 1).

The collagen types synthesized by HISM cells in vitro were identical to those found in strictured bowel, namely types I, III, and V. Production of collagen types with this distribution is characteristic of smooth muscle cells and not fibroblasts (18). The findings demonstrate that HISM cells have the ability to produce the collagen phenotypes that characterize fibrotic lesions of human bowel.

Collagen is a major component of the extracellular matrix and is essential for the structure and strength of all tissues. It is of particular importance when a tissue is injured and rapid repair is required. We speculate, therefore, that HISM cell collagen synthesis is a specialized function that is required to produce additional extracellular matrix in the bowel wall in response to injury. It is also likely that the strictures that complicate inflammatory conditions of the gastrointestinal tract are a fibrotic consequence of this repair process.

References

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